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# A Comparative Study on Invasion, Survival, Modulation of Oxidative Burst, and Nitric Oxide Responses of Macrophages (HD11), and Systemic Infection in Chickens by Prevalent Poultry *Salmonella* Serovars

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**Abstract**

Poultry is a major reservoir for foodborne *Salmonella* serovars. *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Heidelberg, *Salmonella* Kentucky, and *Salmonella* Senftenberg are the most prevalent serovars in U.S. poultry. Information concerning the interactions between different *Salmonella* species and host cells in poultry is lacking. In the present study, the above mentioned *Salmonella* serovars were examined for invasion, intracellular survival, and their ability to modulate oxidative burst and nitric oxide (NO) responses in chicken macrophage HD11 cells. All *Salmonella* serovars demonstrated similar capacity to invade HD11 cells. At 24 h post-infection, a 36–43% reduction of intracellular bacteria, in $\log_{10}$(CFU), was observed for *Salmonella* Typhimurium, *Salmonella* Heidelberg, *Salmonella* Kentucky, and *Salmonella* Senftenberg, whereas a significantly lower reduction (16%) was observed for *Salmonella* Enteritidis, indicating its higher resistance to the killing by HD11 cells. Production of NO was completely diminished in HD11 cells infected with *Salmonella* Typhimurium and *Salmonella* Enteritidis, but remained intact when infected with *Salmonella* Heidelberg, *Salmonella* Kentucky, and *Salmonella* Senftenberg. Phorbol myristate acetate-stimulated oxidative burst in HD11 cells was greatly impaired after infection by each of the five serovars. When newly hatched chickens were challenged orally, a high rate (86–98%) of systemic infection (*Salmonella* positive in liver/spleen) was observed in birds challenged with *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Heidelberg, and *Salmonella* Kentucky, while only 14% of the birds were *Salmonella* Senftenberg positive. However, there was no direct correlation between systemic infection and *in vitro* differential intracellular survival and modulation of NO response among the tested serovars.

## Introduction

*Salmonella* are one of the leading causes of foodborne illness worldwide (Scallan *et al*., 2011). In chickens, infections with host specific serovar *Salmonella* Gallinarum and *Salmonella* Pullorum cause septicemia fowl typhoid and pullorum disease, respectively (Barrow and Freitas Neto, 2011), whereas infections with non-host-specific serovars such as *Salmonella* Typhimurium, *Salmonella* Enteritidis, and *Salmonella* Heidelberg generally display no clinical symptoms. However, these non-host-specific poultry serovars account for the majority of clinical isolates in human salmonellosis. Although *Salmonella* Kentucky and *Salmonella* Senftenberg are not commonly associated with human salmonellosis, they, together with *Salmonella* Typhimurium, *Salmonella* Enteritidis, and *Salmonella* Heidelberg, are the most common serovars isolated from U.S. poultry (CDC, 2008; FDA, 2010). *Salmonella* Senftenberg, a serovar that is more resistant to the environmental stresses, is frequently isolated from hatching houses and raw feed materials, and is adapted to colonize and persist in poultry houses (Liu *et al*., 1969; Bailey *et al*., 2001; Pedersena *et al*., 2008). In the last decade, significant progress has been made in the knowledge of *Salmonella* invasion and pathogenesis in mammalian hosts, most of which are derived from studies based on the murine model of *Salmonella* Typhimurium infection (Haraga *et al*., 2008; Malik-Kale *et al*., 2011). Colonization by *Salmonella* in poultry has been extensively studied and well documented (Foley *et al*., 2011); however, most are epidemiological investigations focused on prevalence. Information regarding *Salmonella* invasion and colonization mechanisms and

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interactions with host cells in chickens is limited and poorly defined (Boyd *et al*., 2007; Lillehoj *et al*., 2007; Chappell *et al*., 2009; Wisner *et al*., 2010, 2011).

Chicken macrophages play a critical role in the defense against microbial infection, in which they detect, phagocytize, and produce microbicidal substances, including reactive radical oxygen species (ROS), nitric oxide (NO), lysozyme, and proteolytic enzymes, to kill the infectious agents (Okamura *et al*., 2005; Withanage *et al*., 2005; Babu *et al*., 2006). Robust macrophage functionality is associated with increased resistance to systemic spread (Wigley *et al*., 2006) and intestinal colonization (Sun *et al*., 2008) by *Salmonella*. However, the role of macrophages in controlling *Salmonella* infection and the interaction between *Salmonella* and macrophages in chickens are much less studied and remain mostly unclear.

In the present study, cell invasion, intracellular survival, and modulation of antimicrobial activity (NO and oxidative burst response) in chicken macrophage HD11 cells of the above mentioned five serovars were examined. Additionally, systemic infection by these five serovars in newly hatched chickens was also investigated.

## Materials and Methods

### *Bacteria*

Primary poultry isolates *Salmonella* Typhimurium and *Salmonella* Enteritidis were obtained from the National Veterinary Services Laboratory (Ames, IA) and were resistant to novobiocin–nalidixic acid (Kogut *et al*., 1995). *Salmonella* Heidelberg, *Salmonella* Kentucky, and *Salmonella* Senftenberg used in this study were field isolates from broilers and were susceptible to novobiocin–nalidixic acid. All strains were susceptible to gentamicin. *Salmonella* from stocks were cultured overnight at 41°C in a Tryptic Soy Broth (TSB; Becton, Dickinson, and Company, Franklin Lakes, NJ) and the overnight cultures were transferred to a fresh TSB and cultured for 4 h to reach an exponential growth phase, and the bacteria were collected, washed, and resuspended in phosphate-buffered saline (PBS) at a final concentration of $\sim 2 \times 10^9$ colony-forming unit (CFU)/mL. Heat-killed *Salmonella* (HKS) were prepared by incubating the bacterial suspension at 75°C water bath for 15 min.

### *Chickens*

Chickens (Hy-Line W36) were obtained from Hy-Line International (Bryan, TX) on the day-of-hatch. Birds were placed in floor pens with pine shavings in a controlled environment (biosafety level 2) and provided *ad libitum* access to water and a balanced unmedicated corn-soybean based diet with nutrient rations meeting or exceeding the recommendations of the National Research Council (NRC, 1994). The experiments comply with the Animal Care and Use Experimental Animal Protocol (Southern Plains Agricultural Research Center, Agriculture Research Service, U.S. Department of Agriculture).

### *HD11 Cells*

The MC29 virus–transformed chicken macrophage cell line HD11 (Beug *et al*., 1979) were maintained in complete Dulbecco's Modified Eagles Medium (DMEM; Invitrogen, Grand Island, NY) containing 10% chicken serum, antibiotics (100 U penicillin/mL and 100 $\mu$g streptomycin/mL), and 1.5 mM L-glutamine (Sigma, St. Louis, MO) at 39°C, 5% $CO_2$, and 95% humidity. Aliquots of cell suspension ($2 \times 10^6$ cells/mL) were seeded into each well at 100 $\mu$L/well for 96-well optical bottom black plate (Nalge Nunc International, Rochester, NY) and 500 $\mu$L/well for 24-well plate (Becton Dickinson Biosciences, San Jose, CA) and allowed to grow to about 85% confluence ($\sim$36 h) before being used for assays. The 96-well plates were used for oxidative burst assay, and the 24-well plates were used for the cell invasion and the NO production assays.

### *Cell invasion and intracellular viability assay*

Prior to infection, the culture medium was removed and cells were washed once and replaced with 200 $\mu$L of plain DMEM (without chicken serum and other additives). Aliquots of 50 $\mu$L of *Salmonella* suspensions ($\sim 2 \times 10^9$ CFU/mL) were added to each well with four replicate wells for each serovar and incubated for 1 h at 39°C in a 5% $CO_2$ humidified incubator. At 1 h post-infection (hpi), the infection medium was removed and the cells were washed once and treated with 100 $\mu$g/mL of gentamicin sulfate in complete DMEM for 1 h to kill extracellular bacteria. After gentamicin treatment, infected cells were washed twice with PBS, lysed for 10 min in 300 $\mu$L of 1% Triton X-100 (in PBS). After lysis, 700 $\mu$L of PBS was added to each well and mixed thoroughly. Serial 1:10 dilutions were spread onto Difco's xylose-lysine tergitol 4 (XLT4) agar (Becton, Dickinson, and Company) plates and incubated at 41°C for 24 h. Colonies were counted to determine the CFU of intracellular bacteria at 2 hpi. Similarly, intracellular viable bacterial CFU was determined at 24 hpi after an additional culture of the infected cells for 22 h in the medium containing 20 $\mu$g/mL of gentamicin sulfate.

### *NO production assay*

Nitrite, a stable metabolite of NO, produced by activated macrophages was measured by the Greiss assay (Green *et al*., 1982). HD11 cells in 24-well plates were treated, in four replicates, with live (prepared in the same way as above) or HKS as described in the killing assay. After 24 h of incubation, nitrite concentrations in the culture media were determined as previously described (He *et al*., 2009).

### *Phorbol myristate acetate (PMA)–stimulated oxidative burst*

Oxidative burst of HD11 cells was measured as described (He *et al*., 2005). To evaluate the effect of *Salmonella* infection on oxidative burst of HD11 cells, PMA (Sigma) stimulated oxidative burst was measured in both *Salmonella*-infected and HKS-treated HD11 cells. The cells were first treated in plain DMEM with 10 $\mu$L of live or HKS suspensions ($\sim 2 \times 10^9$ CFU) in a final volume of 50 $\mu$L/well at 39°C for 1 h. Following the treatment, the cells were washed and stimulated in plain DMEM with PMA (0.5 $\mu$g/mL) in a final volume of 100 $\mu$L/well containing 10 $\mu$g/mL of 2′,7′-dichlorfluorescein-diacetate (DCFH-DA; Sigma) and 100 $\mu$g/mL of gentamicin sulfate for 1 h at 39°C in 5% $CO_2$ and 95% humidity. The relative fluorescent units (RFU) at the end of incubation were measured (485/530 nm) using Genios Plus Plate Reader (Tecan US Inc., Durham, NC).

### In vivo *organ invasion*

Day-old chickens, 25 per group, were orally challenged with 0.5 mL of each different *Salmonella* serovar ($\sim 5 \times 10^8$ CFU/bird) and housed in separated rooms. At 4 days post-infection (dpi), chickens were euthanized with $CO_2$,and liver and spleen were aseptically removed from each chicken and cultured as a combined sample in tetrathionate broth overnight (18–24 h) at 41°C according to guidelines of the U.S. Department of Agriculture (USDA, 1989). After incubation, aliquots of 10 $\mu$L of broth were streaked on XLT4 plates and incubated for 24 h at 41°C. Two independent experiments were conducted at different dates, and a total of 50 chickens were used for each treatment group.

### *Statistical analysis*

At least three independent experiments for NO, oxidative burst, and invasion, and two separate experiments for organ invasion were conducted. Statistical difference was determined at the level of $p < 0.05$ by Student's *t*-test using SigmaStat software (Jandel Corp., Richmond, CA).

## Results

### *Differential modulation of NO production in HD11 cells by* Salmonella *serovars*

*Salmonella* infection–induced NO production in HD11 cells was serovar-dependent (Fig. 1A): *Salmonella* Typhimurium and *Salmonella* Enteritidis induced little or no output of NO, while *Salmonella* Heidelberg, *Salmonella* Kentucky, and *Salmonella* Senftenberg stimulated significant amounts of NO production. This inhibition of NO production in HD11 cells was observed only in treatments with live *Salmonella* Typhimurium and *Salmonella* Enteritidis; HD11 cells stimulated with HKS showed a strong NO production regardless of the *Salmonella* serovar (Fig. 1B).

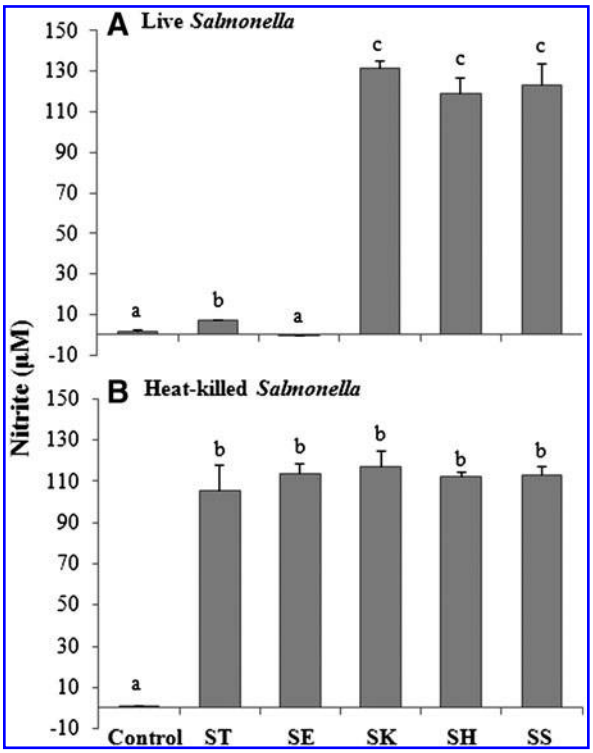


**FIG. 1.** Effect of *Salmonella* infection on nitric oxide (NO) production in HD11 cells. HD11 cells were infected with *Salmonella* for 1 h in 24-well plates at 39°C in a 5% $CO_2$ humidified incubator. At 1 h post-infection (hpi), extracellular *Salmonella* were killed by incubation with media containing 100 $\mu$g/mL of gentamicin sulfate for 1 h; the cells were washed and then cultured for an additional 22 h in a medium containing 20 $\mu$g/mL of gentamicin sulfate; and nitrite contents in cell culture media were determined. Treatment with heat-killed *Salmonella* (HKS) was performed identically as with live *Salmonella*. **(A)** HD11 infected with live *Salmonella*. **(B)** HD11 treated with HKS. ST, *Salmonella* Typhimurium; SE, *Salmonella* Enteritidis; SK, *Salmonella* Kentucky; SH, *Salmonella* Heidelberg; SS, *Salmonella* Senftenberg. Different letters indicate that the difference between these groups is statistically significant ($p < 0.05$).

### *Down-regulation of HD11 cell oxidative burst potential by intracellular* Salmonella

To examine the effect of intracellular *Salmonella* on the oxidative burst capacity of HD11 cells, PMA was used to stimulate oxidative burst in HD11 cells infected with *Salmonella* or treated with HKS. After invasion, all *Salmonella* serovars tested significantly diminished the oxidative burst potential of HD11 cells and rendered the macrophages irresponsive to PMA stimulation (Fig. 2A). Metabolically inhibition by intracellular *Salmonella* was the most likely cause for the loss of oxidative response of *Salmonella*-infected cells to PMA stimulation, since HD11 cells pretreated with HKS in an identical manner displayed no inhibitory effect on oxidative burst response to a subsequent stimulation with PMA (Fig. 2B).

### *Cell invasion and intracellular survival in HD11 cells by* Salmonella

All *Salmonella* serovars demonstrated similar capacity ($p \geq 0.05$) to invade HD11 cells as demonstrated by the CFU at 2 hpi, even though *Salmonella* Typhimurium and *Salmonella* Enteritidis invasion was numerically greater than *Salmonella* Heidelberg, *Salmonella* Kentucky, and *Salmonella* Senftenberg (Table 1). The viability of *Salmonella* Enteritidis at 24 hpi, however, was significantly higher ($p < 0.05$) than that of *Salmonella* Typhimurium, *Salmonella* Heidelberg, *Salmonella* Kentucky, and *Salmonella* Senftenberg, indicating that *Salmonella* Enteritidis was the most resistant to intracellular killing by HD11 cells among the five serovars.

### *Systemic invasion by* Salmonella *in neonatal chickens*

Systemic infection in young chickens by these five *Salmonella* serovars was investigated by examining the presence of *Salmonella* in the liver/spleen of challenged birds at 4 dpi (Table 2). A high percentage (86–98%) of positive birds was observed in groups infected with *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Heidelberg, and *Salmonella* Kentucky, whereas only 14% of the birds were positive in the *Salmonella* Senftenberg challenged group. The unchallenged control birds were all *Salmonella* negative.

## Discussion

Survival inside the macrophage is essential for *Salmonella* virulence and systemic infection (Fields *et al.*, 1986; Schwan

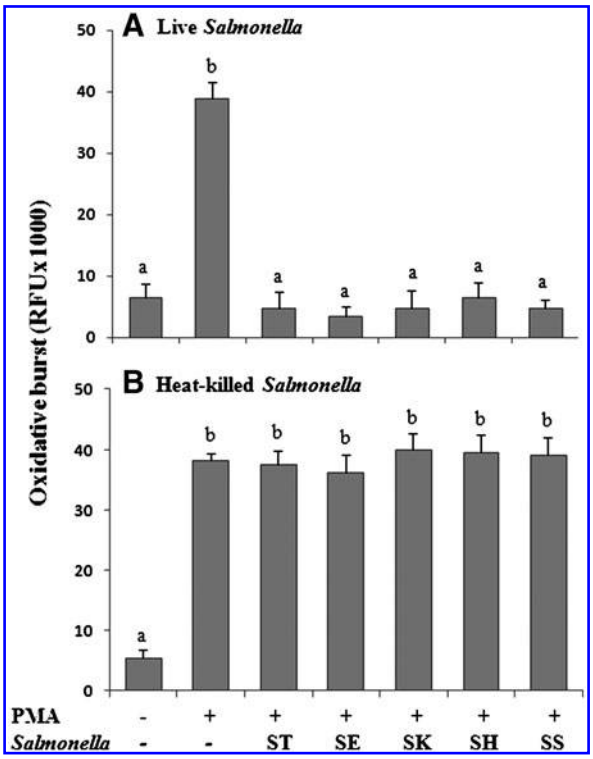


**FIG. 2.** Effect of *Salmonella* infection on Phorbol myristate acetate (PMA)–stimulated oxidative burst in HD11 cells. HD11 cells were infected with *Salmonella* or treated with heat-killed *Salmonella* (HKS) for 1 h in 96-well optical bottom black plates at 39°C in a 5% $CO_2$ humidified incubator. At 1 h post-infection (hpi), the cells were washed and stimulated with PMA (0.5 $\mu$g/mL) in a final volume of 100 $\mu$L/well containing 10 $\mu$g/mL of DCFH-DA and 100 $\mu$g/mL of gentamicin sulfate for 1 h. The relative fluorescent units (RFU) at the end of incubation were measured (485/530 nm). **(A)** HD11 infected with live *Salmonella.* **(B)** HD11 treated with HKS. ST, *Salmonella* Typhimurium; SE, *Salmonella* Enteritidis; SK, *Salmonella* Kentucky; SH, *Salmonella* Heidelberg; SS, *Salmonella* Senftenberg. Different letters indicate that the difference between the groups is statistically significant ($p < 0.05$).

Table 1. Intracellular Survival of *Salmonella* in HD11 Cells[a]

| *Serovar* | *CFU (2 hpi)* | *CFU (24 hpi)* | *Reduction (%)* |
|---|---|---|---|
| *Salmonella* Typhimurium | 6.52 ± 0.47 | 3.63[b] ± 0.40 | 43.29[b] ± 3.79 |
| *Salmonella* Enteritidis | 6.63 ± 0.44 | 5.60[c] ± 0.25 | 16.40[c] ± 6.94 |
| *Salmonella* Kentucky | 5.85 ± 0.51 | 3.74[b] ± 0.78 | 35.98[b] ± 13.32 |
| *Salmonella* Heidelberg | 5.93 ± 0.43 | 3.66[b] ± 0.46 | 37.92[b] ± 4.68 |
| *Salmonella* Senftenberg | 5.95 ± 0.35 | 3.79[b] ± 0.61 | 35.70[b] ± 8.00 |

[a]HD11 cells were infected with *Salmonella* at $2 \times 10^9$ CFU/mL for 1 h at 39°C in a 5% $CO_2$ humidified incubator and intracellular viable *Salmonella* (CFU in log10 scale) at 2 and 24 h post-infection (hpi) were counted. Data are mean ± standard deviations of CFU/well. Reduction (%) = {[CFU (2 hpi) – CFU (24 hpi)]/CFU (2 hpi)}/100. Different letters indicate that the differences between these groups are statistically significant ($p < 0.05$).

Table 2. Organ Invasion by *Salmonella* in Newly Hatched Chickens[a]

| *Serovar* | *Trial 1* | *Trial 2* | *Mean ± SD* |
|---|---|---|---|
| *Salmonella* Typhimurium | 19/25 | 24/25 | 86 ± 14 |
| *Salmonella* Enteritidis | 25/25 | 23/25 | 96 ± 6 |
| *Salmonella* Kentucky | 24/25 | 25/25 | 98 ± 3 |
| *Salmonella* Heidelberg | 24/25 | 24/25 | 96 ± 0 |
| *Salmonella* Senftenberg | 3/25 | 4/25 | 14 ± 3 |

[a]Day-old chickens, 25 per group, were orally challenged with different *Salmonella* serovar (~$5 \times 10^8$ CFU/bird). At 4 days post-infection (dpi), chickens were euthanized, and liver and spleen were removed to test for organ invasion by *Salmonella*. Data in columns for Trials 1 and 2 are *Salmonella*-positive birds in each group at 4 dpi. Data in column for mean ± SD are means and standard deviations of the *Salmonella*-positive birds (%) in each group of the two trials.

*et al*., 2000; Guiney, 2005). *Salmonella* virulence depends at least partially on the type III secretion system (T3SS), which secrets and delivers nearly 40 different virulence effectors into host cells, to facilitate invading, surviving, and replicating within host cells (Haraga *et al*., 2008; Ibarra *et al*., 2009; Malik-Kale *et al*., 2011). To defend against the host cell antimicrobial defense mechanisms, *Salmonella* produce effector proteins which manipulate host cells to delay the phagolysosomal maturation and hence avoid exposure to lysosomal contents (Haraga *et al*., 2008) and secret various metabolic enzymes which neutralize the antimicrobial effect of free radicals oxygen and nitrogen species (ROS and RNS) (Aussel *et al*., 2011; Henard and Vázquez-Torres, 2011; Slauch, 2011).

Professional phagocytes generate ROS in the process of an oxidative burst during phagocytosis of microbes or in response to stimulation by microbial components (Fang, 2011). ROS production in response to microbe and microbial component stimulation play a critical role in controlling microbial infection (Ogier-Denis *et al*., 2008; Lam *et al*., 2010). The exact role of ROS in controlling intracellular *Salmonella* in macrophages is debatable (Fang, 2011), since *Salmonella* carry abundant enzymes (catalases, peroxiredoxins, superoxide dismutases) to neutralize the effect of ROS (Aussel *et al*., 2011; Fang, 2011). PMA is a protein kinase C activator and stimulates a strong oxidative burst in chicken phagocytes (He *et al*., 2005). The effect of intracellular *Salmonella* on the oxidative burst response of HD11 cells to PMA stimulation has not been reported and therefore was examined in the present study. Macrophage HD11 cells infected with the five *Salmonella* serovars showed severely impaired ROS response to PMA stimulation as compared to the non-infected cells, while the cells treated identically with dead *Salmonella* (HKS) demonstrated a normal ROS response to PMA. The lack of ROS response to PMA stimulation in *Salmonella*-infected HD11 cells is likely caused by intracellular *Salmonella* which may inhibit phagocyte NADPH oxidase activity and metabolically neutralize the ROS products. Our results clearly indicate that *Salmonella* serovars are well adapted to evade the ROS-mediated killing in macrophage. Although the ROS-mediated direct killing of intracellular *Salmonella* might be limited in chicken macrophages as our results suggest, accumulated evidence suggests that ROS can act as signaling molecules to indirectly assert an antimicrobial role. For example, ROS have been reported to activate MAP kinase and transcription factors NF-$\kappa$B and AP-1, up-regulate inflammatory cytokine and

chemokine expression, and induce the formation of autophagy (Torres and Forman, 2003; Closa and Folch-Puy, 2004; Huang *et al*., 2011). Activation of these cellular functions plays a critical role in controlling intracellular *Salmonella* (Rosenberger and Finlay, 2002; Sahlberg *et al*., 2007; Jones *et al*., 2008; Deretic, 2011).

NO response to microbial stimulation is an important innate immune function of macrophages and plays a critical role in controlling the proliferation of intracellular bacterial pathogens such as *Salmonella* Typhimurium (Mastroeni *et al*., 2000; Alam *et al*., 2002; 2008). However, virulent factors secreted *via* *Salmonella* T3SS can suppress iNOS activity (Das *et al*., 2009) and prevent iNOS-containing vesicle trafficking to phagosomes, hence limiting exposure of *Salmonella* to RNS (Chakravortty *et al*., 2002). Additionally, *Salmonella* possess three major enzymes (flavohemoglobin Hmp, flavorubredoxin NorV, and cytochrome *c* nitrite reductase NrfA) that can detoxify NO under different environmental conditions (Bang *et al*., 2006; Mills *et al*., 2008). Previously, infection with *Salmonella* Typhimurium and *Salmonella* Enteritidis has been shown to induce NO production in chicken macrophages (Okamura *et al*., 2005; Withanage *et al*., 2005; Babu *et al*., 2006). However, our results show that NO production in HD11 cells was completely inhibited by infection with *Salmonella* Enteritidis and only minor amounts of NO was produced in cells infected with *Salmonella* Typhimurium. Infection of HD11 cells with *Salmonella* Heidelberg, *Salmonella* Kentucky, and *Salmonella* Senftenberg induced large amounts of NO. The results demonstrate a dramatic difference in their ability to modulate host cell NO response among these wild-type *Salmonella* serovars. The lack of or diminished NO response in HD11 cells to *Salmonella* Typhimurium and *Salmonella* Enteritidis infection is probably due to the inhibition on iNOS or the metabolic detoxification of NO, since heat-killed *Salmonella* Typhimurium and *Salmonella* Enteritidis were able to induce large quantities of NO comparable to the levels induced by the other three serovars. The exact mechanism that enables *Salmonella* Typhimurium and *Salmonella* Enteritidis to prevent NO production in HD11 cells is not clear and needs to be further investigated. The discrepancy in NO induction by *Salmonella* Typhimurium and *Salmonella* Enteritidis infection between this study and previous studies (Okamura *et al*., 2005; Withanage *et al*., 2005; Babu *et al*., 2006) cannot be readily explained and may be due to different conditions under which these experiments were conducted.

There was no difference in the rate of internalization of *Salmonella* among the serovars used in this study. Within 24 hpi, HD11 cells were able to limit intracellular *Salmonella* growth and achieved reduction of viable intracellular *Salmonella* of all serovars tested. However, *Salmonella* Enteritidis had the highest viability at 24 hpi among the tested serovars, indicating that *Salmonella* Enteritidis was the most resistant against macrophage-mediated bacterial killing. These results provide supporting evidence to an epidemiological observation that *Salmonella* Enteritidis is more adapted to cause systemic infections in chickens (Foley *et al*., 2011). There was no apparent indication that the ability of *Salmonella* to downregulate ROS and RNS responses provided an advantage in survival within chicken macrophages, since *Salmonella* Typhimurium had a similar, if not the lowest, viability among the five tested serovars despite the fact that it almost completely abrogated NO production in HD11 cells. Therefore, our results indicate that direct killing by ROS or RNS may not play a determinant role in intracellular survival of *Salmonella* in chicken macrophages.

The ability of *Salmonella* to invade *via* the intestine and spread systemically plays an important role in *Salmonella* colonization of reproductive organs, a main cause for internal contamination of eggs (Gast *et al*., 2004). Systemic infection and reproductive organ colonization by serovars *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Salmonella* Heidelberg has been previously reported (Gantois *et al*., 2008). However, the present study was the first to report that *Salmonella* Kentucky is capable of causing systemic infection in liver and spleen through intestinal invasion in young chickens. It is interesting to note that chickens challenged with *Salmonella* Senftenberg had an exceptionally low rate (14%) of systemic infection as compared to chickens challenged with other serovars (86–98%). Although *Salmonella* Senftenberg has been reported to persist in and frequently isolated from poultry hatching houses, farm houses, and raw feed materials (Liu *et al*., 1969; Bailey *et al*., 2001; Kim *et al*., 2007; Pedersena *et al*., 2008), it remains a less prevalent strain in chicks, hens, and poultry products. Our results demonstrated that *Salmonella* Senftenberg lacks the ability to attain systemic infection, suggesting this strain is deficient in its ability to invade.

In summary, we have examined the five most prevalent *Salmonella* serovars in U.S. poultry for intracellular survival and their ability to modulate antimicrobial activity in chicken macrophage HD11 cells. Intracellular *Salmonella* impaired the oxidative burst response of HD11 to PMA stimulation. Infection with *Salmonella* Typhimurium and *Salmonella* Enteritidis, but not *Salmonella* Heidelberg, *Salmonella* Kentucky, and *Salmonella* Senftenberg, abolished NO production in HD11 cells. Serovar *Salmonella* Enteritidis was best adapted to survive inside HD11 cells among the tested serovars. Newly hatched chickens were vulnerable to systemic infection by *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Heidelberg, and *Salmonella* Kentucky, but not *Salmonella* Senftenberg. These results demonstrate the different capabilities to modulate the immune response in chicken macrophages among *Salmonella* serovars; however, further investigations are needed to identify factors that control intracellular survival and systemic infection.

## Disclosure Statement

No competing financial interests exist.

## References

Alam MS, Akaike T, Okamoto S, Kubota T, Yoshitake J, Sawa T, Miyamoto Y, Tamura F, Maeda H. Role of nitric oxide in host defense in murine salmonellosis as a function of its antibacterial and antiapoptotic activities. Infect Immun 2002;70:3130–3142.

Alam MS, Zaki MH, Sawa T, Islam S, Ahmed KA, Fujii S, Okamoto T, Akaike T. Nitric oxide produced in Peyer's patches exhibits antiapoptotic activity contributing to an antimicrobial effect in murine salmonellosis. Microbiol Immunol 2008;52:197–208.

Aussel L, Zhao W, Hébrard M, Guilhon AA, Viala JP, Henri S, Chasson L, Gorvel JP, Barras F, Méresse S. *Salmonella* detoxifying enzymes are sufficient to cope with the host oxidative burst. Mol Microbiol 2011;80:628–640.

Babu US, Gaines DW, Lillehoj H, Raybourne RB. Differential reactive oxygen and nitrogen production and clearance of

*Salmonella* serovars by chicken and mouse macrophages. Dev Comp Immunol 2006;30:942–953.

Bailey JS, Stern NJ, Fedorka-Cray, P, Craven SE, Cox NA, Cosby DE, Ladely S, Musgrove MT. Sources and movement of *Salmonella* through integrated poultry operations: A multistate epidemiological investigation. J Food Prot 2001;64:1690–1697.

Bang IS, Liu L, Vazquez-Torres A, Crouch ML, StamLer JS, Fang FC. Maintenance of nitric oxide and redox homeostasis by the *Salmonella* flavohemoglobin *hmp*. J Biol Chem 2006;281:28039–28047.

Barrow PA, Freitas Neto OC. Pullorum disease and fowl typhoid—New thoughts on old diseases: A review. Avian Pathol 2011;40:1–13.

Beug H, von Kirchbach A, Döderlein G, Conscience JF, Graf T. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell 1979;18:375–390.

Boyd A, Philbin VJ, Smith AL. Conserved and distinct aspects of the avian Toll-like receptor (TLR) system: Implications for transmission and control of bird-borne zoonoses. Biochem Soc Trans 2007;35:1504–1507.

[CDC] Centers for Disease Control and Prevention. *Salmonella* Surveillance: Annual Summary, 2006. Atlanta: CDC, 2008.

Chakravortty D, Hansen-Wester I, Hensel M. *Salmonella* pathogenicity island 2 mediates protection of intracellular *Salmonella* from reactive nitrogen intermediates. J Exp Med 2002;195: 1155–1166.

Chappell L, Kaiser P, Barrow P, Jones MA, Johnston C, Wigley P. The immunobiology of avian systemic salmonellosis. Vet Immunol Immunopathol 2009;128:53–59.

Closa D, Folch-Puy E. Oxygen free radicals and the systemic inflammatory response. IUBMB Life 2004;56:185–191.

Das P, Lahiri A, Lahiri A, Chakravortty D. Novel role of the nitrite transporter NirC in *Salmonella* pathogenesis: SPI2-dependent suppression of inducible nitric oxide synthase in activated macrophages. Microbiol 2009;155:2476–2489.

Deretic V. Autophagy in immunity and cell-autonomous defense against intracellular microbes. Immunol Rev 2011;240:92–104.

Fang FC. Antimicrobial actions of reactive oxygen species. mBio 2011;2:e00141-11. Available at: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3171981/, accessed September 4, 2012.

[FDA] U.S. Food and Drug Administraton. National Antimicrobial Resistance Monitoring System—Enteric bacteria (NARMS): 2007 executive report. Rockville, MD: U.S. Department of Health and Human Services, FDA, 2010.

Fields PI, Swanson RV, Haidaris CG, Heffron F. Mutants of *Salmonella* Typhimurium that cannot survive within the macrophage are avirulent. Proc Natl Acad Sci USA 1986;83: 5189–5193.

Foley SL, Nayak R, Hanning IB, Johnson TJ, Han J, Ricke SC. Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. Appl Environ Microbiol 2011;77:4273–4279.

Gantois I, Eeckhaut V, Pasmans F, Haesebrouck F, Ducatelle R, Van Immerseel F. A comparative study on the pathogenesis of egg contamination by different serotypes of *Salmonella*. Avian Pathol 2008;37:399–406.

Gast RK, Guard-Bouldin J, Holt PS. Colonization of reproductive organs and internal contamination of eggs after experimental infection of laying hens with *Salmonella* Heidelberg and *Salmonella* Enteritidis. Avian Dis 2004;48:863–869.

Green L, Wagner D, Glogowski J, Skipper P, Wishnok J, Tannenbaum S. Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. Anal Biochem 1982;126:131–138.

Guiney DG. The role of host cell death in *Salmonella* infections. Curr Top Microbiol Immunol 2005;289:131–150.

Haraga A, Ohlson MB, Miller SI. Salmonellae interplay with host cells. Nat Rev Microbiol 2008;6:53–66.

He H, Lowry VK, Swaggerty CL, Ferro PJ, Kogut MH. *In vitro* activation of chicken leukocytes and *in vivo* protection against *Salmonella enteritidis* organ invasion and peritoneal *S. enteritidis* infection–induced mortality in neonatal chickens by immunostimulatory CpG oligodeoxynucleotide. FEMS Immunol Med Microbiol 2005;43:81–89.

He H, MacKinnon KM, Genovese KJ, Nerren JR, Swaggerty CL, Nisbet DJ, Kogut MH. Chicken scavenger receptors and their ligand-induced cellular immune responses. Mol Immunol 2009;46:2218–2225.

Henard CA, Vázquez-Torres A. Nitric oxide and *Salmonella* pathogenesis. Front Microbiol 2011;2:84.

Huang J, Lam GY, Brumell JH. Autophagy signaling through reactive oxygen species. Antioxid Redox Signal 2011;14:2215–2231.

Ibarra JA, Steele-Mortimer O. *Salmonella*—The ultimate insider. *Salmonella* virulence factors that modulate intracellular survival. Cell Microbiol 2009;11:1579–1586.

Jones RM, Wu H, Wentworth C, Luo L, Collier-Hyams L, Neish AS. *Salmonella* AvrA coordinates suppression of host immune and apoptotic defenses via JNK pathway blockade. Cell Host Microbe 2008;3:233–244.

Kim A, Lee YJ, Kang MS, Kwag SI, Cho JK. Dissemination and tracking of *Salmonella* spp. in integrated broiler operation. J Vet Sci 2007;8:155–161.

Kogut MH, McGruder ED, Hargis BM, Corrier DE, DeLoach JR. *In vivo* activation of heterophil function in chickens following injection with *Salmonella enteritidis*–immune lymphokines. J Leukoc Biol 1995;57:56–62.

Lam GY, Huang J, Brumell JH. The many roles of NOX2 NADPH oxidase-derived ROS in immunity. Semin Immunopathol 2010; 32:415–430.

Lillehoj HS, Kim CH, Keeler CL Jr, Zhang S. Immunogenomic approaches to study host immunity to enteric pathogens. Poult Sci 2007;86:1491–1500.

Liu TS, Snoeyenbos GH, Carlson VL. Thermal resistance of *Salmonella* Senftenberg 775W in dry animal feeds. Avian Dis 1969;13:611–631.

Malik-Kale P, Jolly CE, Lathrop S, Winfree S, Luterbach C, Steele-Mortimer O. *Salmonella*—At home in the host cell. Front Microbiol 2011;2:125.

Mills PC, Rowley G, Spiro S, Hinton JC, Richardson DJ. A combination of cytochrome c nitrite reductase (NrfA) and flavorubredoxin (NorV) protects *Salmonella enterica* serovar Typhimurium against killing by NO in anoxic environments. Microbiology 2008;154:1218–1228.

Mastroeni P, Vazquez-Torres A, Fang FC, Xu Y, Khan S, Hormaeche CE, Dougan G. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. II. Effects on microbial proliferation and host survival *in vivo*. J Exp Med 2000;192:237–248.

[NRC] National Research Council. *Nutrient Requirements of Poultry*, 9th rev. ed. Washington, DC: Academic Press, 1994.

Okamura M, Lillehoj HS, Raybourne RB, Babu US, Heckert RA, Tani H, Sasai K, Baba E, Lillehoj EP. Differential responses of macrophages to *Salmonella enterica* serovars Enteritidis and Typhimurium. Vet Immunol Immunopathol 2005;107:327–335.

Ogier-Denis E, Mkaddem SB, Vandewalle A. NOX enzymes and Toll-like receptor signaling. Semin Immunopathol 2008;30: 291–300.

Pedersena TB, Olsena JE, Bisgaarda M. Persistence of *Salmonella* Senftenberg in poultry production environments and investigation of its resistance to desiccation. J Avian Pathol 2008; 37:421–427.

Rosenberger CM, Finlay BB. Macrophages inhibit *Salmonella* Typhimurium replication through MEK/ERK kinase and phagocyte NADPH oxidase activities. J Biol Chem 2002;277: 18753–18762.

Sahlberg AS, Penttinen MA, Heiskanen KM, Colbert RA, Sistonen L, Granfors K. Evidence that the p38 MAP kinase pathway is dysregulated in HLA-B27-expressing human monocytic cells: Correlation with HLA-B27 misfolding. Arthritis Rheum 2007;56:2652–2662.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, Jones JL, Griffin PM. Foodborne illness acquired in the United States—Major pathogens. Emerg Infect Dis 2011;17:7–15.

Schwan WR, Huang XZ, Hu L, Kopecko DJ. Differential bacterial survival, replication, and apoptosis-inducing ability of *Salmonella* serovars within human and murine macrophages. Infect Immun 2000;68:1005–1013.

Slauch JM. How does the oxidative burst of macrophages kill bacteria? Still an open question. Mol Microbiol 2011;80:580–583.

Sun SF, Pan QZ, Hui X, Zhang BL, Wu HM, Li H, Xu W, Zhang Q, Li JY, Deng XM, Chen JW, Lian ZX, Li N. Stronger *in vitro* phagocytosis by monocytes-macrophages is indicative of greater pathogen clearance and antibody levels *in vivo.* Poult Sci 2008;87:1725–1733.

Torres M, Forman HJ. Redox signaling and the MAP kinase pathways. Biofactors 2003;17:287–296.

[USDA] U.S. Department of Agriculture, Animal and Plant Health Inspection Service. *National Poultry Improvement Plan and Auxiliary Provision.* Veterinary Services, Publication APHIS, Washington, DC: U.S. Government Printing Office, 1989, pp. 40–91.

Wigley P, Hulme S, Rothwell L, Bumstead N, Kaiser P, Barrow P. Macrophages isolated from chickens genetically resistant or susceptible to systemic salmonellosis show magnitudinal and temporal differential expression of cytokines and chemokines following *Salmonella enterica* challenge. Infect Immun 2006;74: 1425–1430.

Wisner AL, Desin TS, Koch B, Lam PK, Berberov EM, Mickael CS, Potter AA, Köster W. *Salmonella enterica* subspecies *enterica* serovar Enteritidis *Salmonella* pathogenicity island 2 type III secretion system: Role in intestinal colonization of chickens and systemic spread. Microbiology 2010;156:2770–2781.

Wisner AL, Potter AA, Köster W. Effect of the *Salmonella* pathogenicity island 2 type III secretion system on *Salmonella* survival in activated chicken macrophage-like HD11 cells. PLoS One 2011;6:e29787.

Withanage GS, Mastroeni P, Brooks HJ, Maskell DJ, McConnell I. Oxidative and nitrosative responses of the chicken macrophage cell line MQ-NCSU to experimental *Salmonella* infection. Br Poult Sci 2005;46:261–267.

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